MnmA and IscS Are Required for in Vitro 2-Thiouridine Biosynthesis in *Escherichia coli*†

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ABSTRACT: Thionucleosides are uniquely present in tRNA. In many organisms, tRNA specific for Lys, Glu, and Gln contain hypermodified 2-thiouridine (s^2U) derivatives at wobble position 34. The s^2 group of s^2U34 stabilizes anticodon structure, confers ribosome binding ability to tRNA and improves reading frame maintenance. Earlier studies have mapped and later identified the *mnmA* gene (formerly *asuE* or *trmU*) as required for the s^2U modification in *Escherichia coli*. We have prepared a nonpolar deletion of the *mnmA* gene and show that it is not required for viability in *E. coli*. We also cloned *mnmA* from *E. coli*, and overproduced and purified the protein. Using a gel mobility shift assay, we show that MnmA binds to unmodified *E. coli* $tRNA^{Lys}$ with affinity in the low micromolar range. MnmA does not bind observably to the nonsubstrate *E. coli* $tRNA^{Phe}$. Corroborating this, $tRNA^{Glu}$ protected MnmA from tryptic digestion. ATP also protected MnmA from trypsinolysis, suggesting the presence of an ATP binding site that is consistent with analysis of the amino acid sequence. We have reconstituted the in vitro biosynthesis of s^2U using unmodified *E. coli* $tRNA^{Glu}$ as a substrate. The activity requires MnmA, Mg-ATP, L-cysteine, and the cysteine desulfurase IscS. HPLC analysis of thiolated tRNA digests using [^{35}S]cysteine confirms that the product of the in vitro reaction is s^2U. As in the case of 4-thiouridine synthesis, purified IscS-persulfide is able to provide sulfur for in vitro s^2U synthesis in the absence of cysteine. Small RNAs that represent the anticodon stem loops for $tRNA^{Glu}$ and $tRNA^{Lys}$ are substrates of comparable activity to the full length tRNAs, indicating that the major determinants for substrate recognition are contained within this region.

Nucleoside modifications are introduced posttranscriptionally into RNAs of all organisms (*1*). Sulfur modification of nucleosides is also common but has been observed only in transfer RNA. The uridine at wobble position 34 in $tRNA^{Lys}$, $tRNA^{Glu}$, and $tRNA^{Gln}$ from many species contains sulfur at the 2-position (s^2). The 2-thiouridine (s^2U)[1] at this position is further modified to 5-methylaminomethyl-2-thiouridine (mnm^5s^2U) and 5-methoxycarbonylmethyl-2-thiouridine (mcm^5s^2U) in *Escherichia coli* and humans, respectively. Structurally, the sulfur at position 2 and hypermodification at position 5 act to stabilize the C3′-*endo* conformation of the ribose ring (*2*, *3*). This increases conformational rigidity and minimizes wobble base pairing with guanosine at the third position of the codons in vitro (*4*). Together with the hypermodified A37, these s^2U derivatives are responsible for stabilizing the canonical loop structure in the anticodon domain of $tRNA^{Lys}$ (*5*).

In addition to its unique conformational effects, the s^2U modification is required for many of the biochemical functions of tRNAs in which it is found. For example, the s^2U in *E. coli* $tRNA^{Glu}$ is a recognition element for glutamyl-aminoacyl tRNA synthetase (*6*−*8*). The s^2U in *E. coli* $tRNA^{Glu}$ also appears to primarily increase the translation rate of GAA codons in vivo (*9*). Mutants lacking s^2U display a significant increase in frameshifting, a trait common to many tRNA modification mutants (*10*). Thiolation at position 2 is also essential for *E. coli* $tRNA^{Lys}_{mnm5s2UUU}$ to interact with AAA or AAG programmed ribosomes (*11*).

The s^2U modification has recently been implicated in some novel aspects of human disease. It is a recognition element for the efficient use of human $tRNA^{Lys,3}$ as a primer for HIV reverse transcriptase (*12*, *13*). A point mutation in a mitochondrial $tRNA^{Lys}$ gene (A8344G) responsible for myoclonus epilepsy associated with ragged-red fibers (MERRF) leads to lack of s^2U modification in the hypermodified wobble nucleotide (*14*). This mutation was demonstrated to cause severe impairment of mitochondrial protein synthesis and respiratory deficiency in MERRF patients (*15*). The unmodified wobble $tRNA^{Lys}$ mutant did not bind mRNA programmed 30S ribosomes (*16*), consistent with similar findings in *E. coli* (*11*, *17*).

The known enzymatic steps involved in the biosynthesis of mnm^5s^2U34 are outlined in Figure 1. Sullivan et al. (*18*) reported an *E. coli asuE* mutant defective in the 2-thiolation step in the biosynthesis of mnm^5s^2U34. Consistent with this, mnm^5U34 was later identified in total tRNA of the *asuE* mutant strain (*19*). The *asuE* or *trmU* gene is currently renamed *mnmA* (*20*). Elseviers et al. (*21*) isolated an *E. coli trmE* (now *mnmE*) mutant containing s^2U34, suggesting the *mnmE* gene product catalyzes one of the steps involved in the synthesis of $cmnm^5s^2U34$ at the 5 position. It is not

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[1] Abbreviations: s^2U, 2-thiouridine; s^4U, 4-thiouridine; mnm^5s^2U, 5-methylaminomethyl-2-thiouridine; PLP, pyridoxal 5-phosphate; DTT, dithiothreitol; IPTG, iso-propyl β-D-thiogalactopyranoside.

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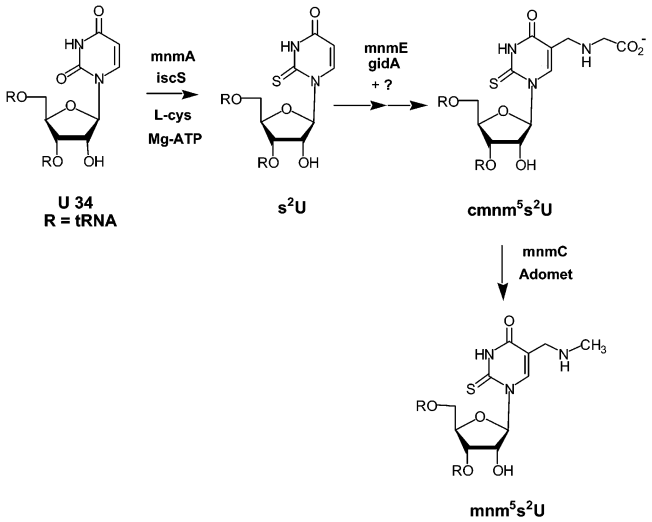

FIGURE 1: Biosynthesis of mnm^5s^2U in *E. coli*.

known how many steps are involved in the conversion of s^2U34 to $cmnm^5s^2U34$. Recently, Bregeon et al. (*22*) proposed that GidA and MnmE participate in the same tRNA modification pathway, whose absence results in a +2 ribosomal frame shift. The isolation of mutants containing either s^2 or mnm^5 modifications only on the uridine 34 suggests that, these modifications occur independently (*18*, *15*). Finally, the $cmnm^5s^2U$ is first demodifed to nm^5s^2U and later methylated in a S-adenosyl-methionine dependent step (*23*). The last two steps are catalyzed by the same enzyme, MnmC (*23*, *24*).

To characterize the enzymatic pathway of s^2U biosynthesis, we have cloned *mnmA* from *E. coli*, purified the protein and checked its ability to bind tRNA. We have also attempted to reconstitute s^2U biosynthesis in vitro with MnmA and IscS, a cysteine desulfurase that we have previously shown to be a sulfurtransferase for 4-thiouridine (s^4U) synthesis (*25*, *27*). We and others have recently reported that *iscS* is required for s^2U modification in *E. coli* (*38*, *39*) and *S. typhimurium* (*40*). In this paper, we report evidence that MnmA binds specifically to its substrate tRNAs over nonsubstrate $tRNA^{Phe}$ and catalyzes s^2U synthesis in vitro utilizing sulfur mobilized by IscS.

MATERIALS AND METHODS

Materials. L-[^{35}S]Cysteine (1075 Ci/mmol) and [γ^{32}P]ATP (6000 Ci/mmol) were purchased from NEN Life Sciences Products. Phenyl-agarose, Reactive Green 19-agarose, TPCK-trypsin, wide molecular weight range protein standards, and bacterial alkaline phosphatase (type III) were purchased from Sigma. Nuclease P1 was purchased from Roche Biosciences and DEAE-Sepharose Fast Flow was purchased from Pharmacia Biotech. DE81 filter disks are from Whatman. DNA oligos were purchased from Integrated DNA Technology and further purified by denaturing PAGE before use. Strain PAR1219 containing the expression plasmid for T7 RNA polymerase was kindly provided by Dr. F. W. Studier, Biology Department, Brookhaven National Laboratory.

In Frame Deletion of mnmA in E. coli MC1061. The gene deletion protocol was that of Link et al. (*26*), and was previously described by us for the deletion of *iscS* in the same parent strain (*27*). PCR analysis was used to confirm the absence of the gene and the presence of a 36 bp replacement allele (See Table 1).

Cloning of MnmA. The *E. coli mnmA* gene is designated as *ycfB* in the *E. coli* genome submission (accession number AE000213). However, the N-terminal sequence annotated in this submission differs from the N-terminus reported in the SwissProt database (accession no. P25745). Thus, we altered the primer sequence accordingly to express the correct N-terminal sequence which begins MSETAKKV... The primers (Table 1) were used to amplify the *mnmA* gene from *E. coli* K-12 genomic DNA with flanking *Nde*I and *Bam*HI restriction sites. After digestion, the insert was ligated into similarly digested pET-21c to create plasmid pCL250. This plasmid was used to transform BL21(DE3) cells (Novagen) for the overexpression of MnmA. A separate plasmid (pCL250His) was constructed with a His_6 fusion at the N-terminus of MnmA using the His-mnmA primers (Table 1). Both plasmids complement the loss of mnm^5s^2U in the tRNA of our *mnmA* deletion mutant.

Purification of MnmA. A 1% overnight culture of cells carrying *mnmA* plasmid pCL250 was used to inoculate 4.0 L of LB medium containing ampicillin (100 μg/mL) and grown to an A_{600} of 0.6. Expression was induced by adding IPTG to a final concentration of 0.3 mM and growth continued for 3 h. Cells were harvested and stored at −70 °C. PMSF (1.0 mL of 0.2 M solution in *n*-propanol), 2-mercaptoethanol (1 mL), and glycerol (10% final concentration) were added routinely to 1 L of all buffers used in the purification of MnmA. All steps were carried out at 0 to 4 °C. The frozen cell pellet from 4.0 L of culture (10 g) was resuspended in 64 mL of 50 mM Tris/HCl, pH 7.5, 200 mM NaCl, 5% glycerol, 1 mM DTT, 1 mM PMSF, and treated with lysozyme (0.3 mg/mL, 1 h, 0 °C). DNAse I (10 μg) was added and further incubated for 30 min at 0 °C. The S-30 lysate was centrifuged at 100000*g* for 4.0 h. The S-100 supernatant was diluted by adding 3 vol of 10 mM Tris/HCl, pH 7.5, and applied to a DEAE-Sepharose Fast Flow column (50 mL of settled gel) equilibrated with 10 mM Tris/HCl, pH 7.5, 50 mM KCl. The column was washed with 100 mL of equilibration buffer containing 125 mM KCl. Bound protein was eluted with a linear salt gradient (125−500 mM KCl) in 500 mL of equilibration buffer. Fractions containing MnmA were pooled, diluted with 1 vol of 10 mM Tris/HCl, pH 7.5, and applied to a Reactive Green 19-agarose column (25 mL of settled gel) equilibrated with 10 mM Tris/HCl, pH 7.5, 100 mM KCl. The column was eluted with a KCl gradient (100−750 mM KCl) in 250 mL of equilibration buffer. Peak fractions having MnmA protein were pooled. Solid ammonium sulfate was added to the pooled MnmA protein to give a final concentration of 1.75 M and applied to a Phenyl-agarose column (40 mL of settled gel), equilibrated with 10 mM Tris/HCl, pH 7.5, 1.75 M ammonium sulfate. The column was eluted with a reverse ammonium sulfate gradient (1.75−0.0 M) in 400 mL of equilibration buffer. Peak fractions with MnmA protein were pooled and concentrated using Centricon YM-10 devices.

Preparation of tRNA Transcripts. Unmodified *E. coli* $tRNA^{Phe}$ was prepared by in vitro transcription of *Bst*N1-digested plasmid DNA (pCF23) as described (*28*). Small RNA stem loop mimics were prepared by runoff transcription of synthetic single strand oligos. $tRNA^{Glu}$, $tRNA^{Glu}$U8C, $tRNA^{Glu}$U34A, $tRNA^{Lys}$, $tRNA^{Lys}$U8C transcripts used in the

Table 1: Strains, Plasmids, and Primers Used in This Work[a]

strains	genotype
MC1061	F^- araD139 Δ(ara leu)7696 Δ(lacY74) galU galK hsdr $hsdM^+$ strA
CL250 (ΔmnmA)	F^- araD139 Δ(ara leu)7696 Δ(lacY74) galU galK hsdr $hsdM^+$ ΔmnmA strA
plasmids	**description**
pCL250	pET21c derivative containing wt E. coli mnmA
pCL250His	pET15b derivative containing wt mnmA with a N-terminal His_6 tag
primers	**sequence**
mnmA-No	5′AAGGAAAAAAGCGGCCGCACCGTTGGCTGATATTTCCGA
mnmA-Ni	5′CACGCAATAACCTTCACACTCCAAATTTATAACCACTCAATGCAA GGAATCAGGCT
mnmA-Ci	5′GTTATAAATTTGGAGTGTGAAGGTTATTGCGTGTAATCGGTATCG GAATCAGGAGAATTTATAATGG
mnmA-Co	5′-CGCACGCATGTCGACACCCTTTACCGCGGTTAGC
MnmA-5′	5′-TAA AGA CAT ATG TCT GAA ACC GCA AAA AAA GTA
MnmA-3′	5′-GAT GGA TCC TCA GAC CGG CAG CGG CAG ACG
HisMnmA-5′	5′- AGT GAT CAT ATG TCT GAA ACC GCA AAA AAA GTA
HisMnmA-3′	5′- GAT GGA TCC GAG ACC GGC AGC GGC AGA CGC TG

[a] For primers, restriction sites are underlined and the 33 bp gene replacement tag sequence is shown in bold (see Materials and Methods).

present study were prepared by runoff transcription with T7 RNA polymerase using double-stranded DNA templates prepared as described (*29*). Two complementary oligonucleotides with appropriate substitutions were designed. One strand of oligonucleotide contains T7 promoter followed by the first half of the tRNA gene sequence, and the second strand contains the other half of the complementary sequence of the gene including a 12 bp overlapping region. A list of synthetic DNA oligonucleotides used in the generation of tRNA transcripts is given in Table 1. The two oligonucleotide strands were annealed and extended by reverse transcription (*29*) except that the reaction mixtures were incubated overnight. The resulting double-stranded DNA templates were phenol-extracted, ethanol-precipitated, and used as a substrate in the transcription of tRNA. The resulting tRNA was purified by electrophoresis on 20% denaturing polyacrylamide gels. tRNA in the gel was detected by UV shadowing and the tRNA band was cut, crushed, and incubated with 0.5 M NaCl for 4.0 h to overnight. The eluted tRNA was phenol-extracted, and precipitated with ethanol. The tRNA precipitate was dissolved in 5 mM $MgCl_2$, denatured at 65 °C for 5 min, and then renatured by slow cooling to room temperature (~20 min).

Agarose Gel Mobility Shift. MnmA (6.0 μM) was incubated with varying amounts (0−96 μM) of $tRNA^{Lys}$U8C in 25 μL of 20 mM Tris-acetate, pH 7.6, 5 mM $MgCl_2$ for 2 min at 37 °C. Samples were then applied to 2% native agarose gels, and electrophoresis was performed as described below.

Zone-Interference Electrophoresis. Zone-interference electrophoresis was performed as described by Abrahams and co-workers (*30*) on vertical agarose gels. Electrophoresis was performed on 1.5 mm, 2% w/v agarose gels employing a Bio-Rad minigel apparatus at 200 V in cold room for 30 min. The gel and electrophoresis buffer contained 20 mM Tris-acetate (pH 7.6) and 5 mM $MgCl_2$. MnmA (32 μM) was incubated with $tRNA^{Lys}$ U8C (32 μM) in 5 μL above buffer containing 10% glycerol for 2 min at 37 °C. Samples were then applied beneath a series of zone solutions of $tRNA^{Lys}$U8C (50 μL) of (0, 0.5, 1, 2, 4, 6, 8 μM in the same buffer containing 4% glycerol) and electrophoresed. The gels were stained with Coomassie stain to visualize proteins.

Proteolytic Cleavage of MnmA with Trypsin. MnmA (0.13 mg/mL) in 50 mM Tris/HCl, pH 7.5, 50 mM KCl, 10 mM $Mg(OAc)_2$ was digested at 37 °C with TPCK-trypsin at a MnmA/trypsin ratio of 100:1 (w/w). In the protection experiments, MnmA was preincubated with tRNA transcripts (at a molar ratio of 1:5) or 1 mM nucleotides (ATP or GTP) at 37 °C for 5 min, and then reaction mixtures were digested as above. Aliquots containing 2.0 μg of MnmA digest were removed at various time intervals as indicated in the figure legends and analyzed by electrophoresis on 10% SDS−polyacrylamide gels. Proteins were visualized by Coomassie staining.

In Vitro tRNA Thiolation Assay. For purification of MnmA, reactions were carried out in a total volume of 50 μL containing 10 mM Tris/HCl, pH 7.5, 50 mM KCl, 12 mM $Mg(OAc)_2$, 1 mM ATP, 5 μg of $tRNA^{Glu}$U8C transcript, 10 μM [^{35}S]cysteine, 20 μM PLP, 0.1 mM DTT, 1.4−2.0 μg of IscS, and 5−10 μg of purified overexpressed MnmA or 25−50 μg of crude *E. coli* MnmA overexpressed S-30/S-100. The reaction mixtures were incubated at 37 °C for 10 min and applied to DEAE 81 filters and processed by following a modified method of Harris and Kolanko (*31*), which we have described in detail elsewhere (*25*). While full-length tRNAs were folded using a slow cooling protocol, small stem loop substrates were heated at 95 °C for 5 min in 10 mM Tris, pH 7.5, 0.5 mM EDTA, and 7 mM $MgCl_2$, then placed immediately on ice. This procedure was found by Soderberg and Poulter (*32*) to increase monomeric stem loop formation in $tRNA^{Phe}$ derived stem loops. For kinetic analysis, 10 μg (5 μM) of purified MnmA and 3.5 μg (1.5 μM) of IscS were used in a 50 μL reaction mixture containing identical concentrations of Tris and salts as above, but with 4 mM ATP, 20 μM [^{35}S]cysteine, 1 mM DTT, and no PLP. Reaction times were 10 min at 37 °C and reactions were analyzed for ^{35}S incorporation by application to filter disks as above.

Isolation of ^{35}S-Labeled tRNA. Large-scale tRNA thiolation reactions were carried out in a volume of 0.6−2.5 mL as described above except that the reaction was terminated by ethanol precipitation. The labeled tRNA precipitate was dissolved in water and purified by denaturing PAGE (20% gels).

Digestion of tRNA and HPLC Analysis of Nucleosides. Digestion of tRNA transcript was performed with nuclease

P1 followed by bacterial alkaline phosphatase essentially as described by Gherke et al. (*33*). Reaction mixtures containing either native or thiolated tRNA transcripts (37−144 μg) in 200 μL of 30 mM NaOAc, pH 5.3, 0.2 mM $ZnSO_4$ were incubated with 52 μg of nuclease P1 at 37 °C for 2 h. To this mixture, 20 μL of 1.0 M Tris/HCl, pH 8.3 was added followed by 7.8 units of calf intestinal alkaline phosphatase and incubated for >1 h. Aliquots of 20 μg of digested tRNA were analyzed by reversed-phase HPLC using Supelco LC-18S column. Because of the difficulty in separating s^2U from guanosine, the following modified buffer conditions were used. Buffer A was increased to 4% methanol in 10 mM ammonium phosphate pH 5.3 and buffer B was 20% methanol in 10 mM ammonium phosphate pH 5.3. The stepwise gradient program of Gherke et al. (*33*) was used for their high-resolution separation of modified nucleosides. For ^{35}S analysis, 1 mL fractions were collected and aliquots of 0.1 mL were added to scintillation cocktail and counted using a Packard Tricarb liquid scintillation counter.

Assay for IscS Persulfide Activity in the in Vitro Synthesis of s^2U. Reactions were carried out exactly as described for in vitro tRNA thiolation assay except [^{35}S]cysteine was omitted and IscS-^{35}S persulfide was used in the place of IscS. IscS persulfide was formed by reaction of IscS with ^{35}S-L-cysteine for 2 min and purified on a Sephadex G-50 column to remove excess cysteine. Reactions were performed either in the presence or absence of 0.1 mM DTT. IscS-^{35}S persulfide containing 0.2 mol of bound sulfur/mol of protein was used in the assay. Reactions containing 10 μg (5 μM) of MnmA, 5 μg (4 μM) of tRNA transcript, and 10 μg (4.4 μM) of IscS-^{35}S persulfide were incubated at 37 °C for 30 min and subjected to electrophoresis on 8% denatured PAGE gels. Labeled bands were visualized by PhosphorImager analysis (Molecular Dynamics).

RESULTS

Deletion of MnmA in E. coli MC1061. E. coli mnmA mutants have been described previously (*18*, *19*). Since these mutants are not deletions and have a slow growth phenotype, the possibility remained that *mnmA* was required for viability in *E. coli*. We prepared an in-frame deletion of the *mnmA* gene in *E. coli* MC1061 using the gene replacement method of Link et al. (*26*) to solve this issue and for analysis of *mnmA* expression plasmids. The resulting mutant strain, CL250, has a slow growth phenotype in rich medium similar to that previously described for other *mnmA* point mutants (*19*). The viability of the *mnmA* deletion strain shows that *mnmA* is not an essential gene in *E. coli*. We also confirmed the presence of mnm^5U and complete absence of mnm^5s^2U in tRNA isolated from the deletion strain by HPLC analysis (Figure 2). Using strain CL250(DE3), a DE3 lysogen, we have found that the *mnmA* T7-based expression plasmids pCL250 and pCL250His fully complement this mutant with respect to growth and the level of mnm^5s^2U in the tRNA (Figure 2, panel C). This allowed us to pursue with confidence the use of purified recombinant MnmA for the synthesis of s^2U in vitro.

Purification of Overexpressed MnmA. We cloned *mnmA* from *E. coli* K-12 and overexpressed in strain BL21(DE3) as described in the Materials and Methods. The overproduced MnmA, which ran at ~45 kDa on SDS gels was purified

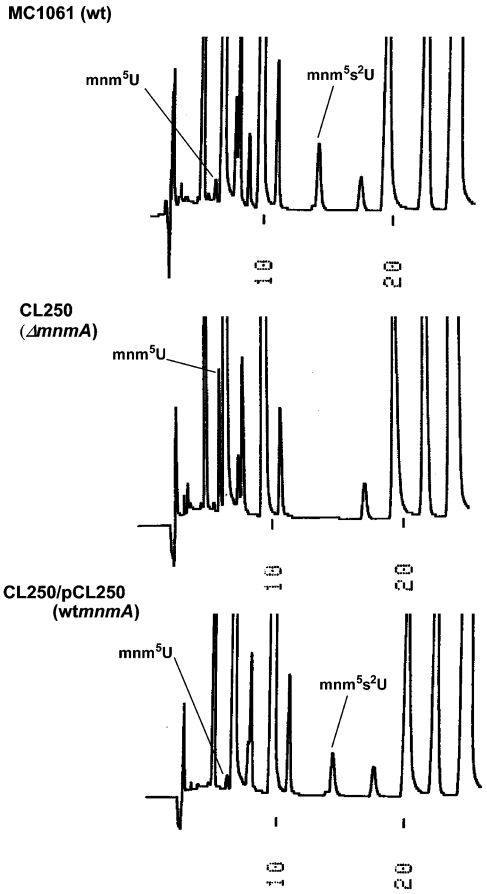

FIGURE 2: HPLC analysis of tRNA isolated from *mnmA* deletion strain. Panels A−C represent chromatograms of tRNA digests from *E. coli* parent strain MC1061, *mnmA*$^-$ strain CL250, and *mnmA*$^-$ strain complemented with *mnmA* expression plasmid pCL250, respectively.

sequentially on DEAE-Sepharose, reactive green-19-agarose, and Phenyl-agarose columns. Quantitative analysis of the MnmA after Phenyl-agarose step established a protein purity of at least 91.6%.

tRNA Binding Studies of MnmA by Protease Protection Assay. We first assessed the ability of MnmA to bind unmodified tRNAs using a protease protection assay (Figure 3). Treatment of MnmA with TPCK-trypsin under mild conditions (100: 1 w/w) generated two small peptides of estimated molecular mass 20 kDa (panel A). MnmA was completely degraded at the end of 10 min of digestion. The two peptides generated were also completely degraded after 20 min of digestion. We looked for tRNA binding and protection of MnmA from proteolytic cleavage (panel B−D). When MnmA was digested in the presence of substrate tRNAs, $tRNA^{Glu}$ and $tRNA^{Lys}$, much of the MnmA remained intact during the time course (30 min) of digestion (panels B and C). The specificity of this protection was verified by digesting MnmA in the presence of nonsubstrate tRNA, $tRNA^{Phe}$. Data presented in Figure 3, panel D, demonstrate that MnmA was completely degraded in the presence of $tRNA^{Phe}$ and the digestion pattern was identical to the one shown in panel A, where MnmA was digested alone. Thus, MnmA appears to bind its substrate tRNAs, unmodified $tRNA^{Glu}$ and $tRNA^{Lys}$.

ATP Protects MnmA from Proteolysis. MnmA contains a nucleotide binding loop similar to ThiI and other enzymes

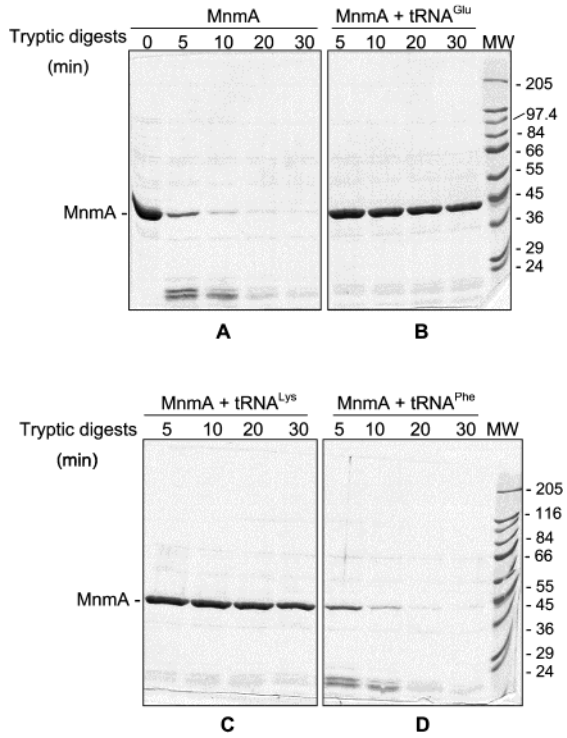

FIGURE 3: Protection of tryptic cleavage of MnmA by substrate tRNA transcripts. MnmA was digested with trypsin in the presence and absence of tRNA transcript's as described in the Materials and Methods. Panels A−D represent digests of MnmA alone, MnmA + tRNA^{Glu}U8C, tRNA^{Lys}U8C, and tRNA^{Phe}, respectively. Aliquots of MnmA digests at 0, 5, 10, 20, and 30 min were analyzed by SDS−PAGE. Lane MW, standard protein markers.

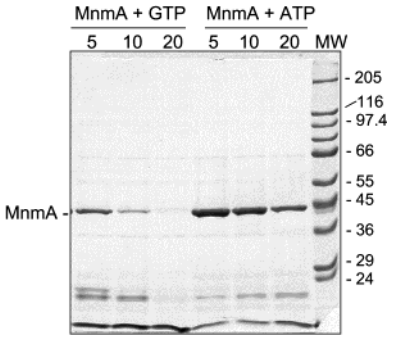

FIGURE 4: ATP and not GTP protects MnmA from trypsinolysis. MnmA was digested with trypsin in the presence of either ATP or GTP as described in the Materials and Methods. MnmA digests at 5, 10, and 20 min were analyzed by SDS−PAGE.

that use NTPs for activation. The usual sources of such activation are ATP or GTP. To determine which is required for MnmA activation of U34, we have performed comparative proteolysis of MnmA with trypsin in the presence of either ATP or GTP (Figure 4). MnmA was degraded completely in the presence GTP and the digestion pattern was identical to the protein digested alone as shown in Figure 3, panel A. Whereas, MnmA remained intact at the end of 10 min of digestion in the presence of ATP and thereafter rate of proteolysis was slowed. This protective effect of ATP on MnmA digestion is evidence that MnmA binds ATP which is required for s^2U synthesis.

tRNA Binding Studies of MnmA by Gel Mobility Shift Assay. We have investigated the affinity of MnmA to tRNA using a variety of techniques. Methods that employed variable MnmA concentration were not reproducible in our

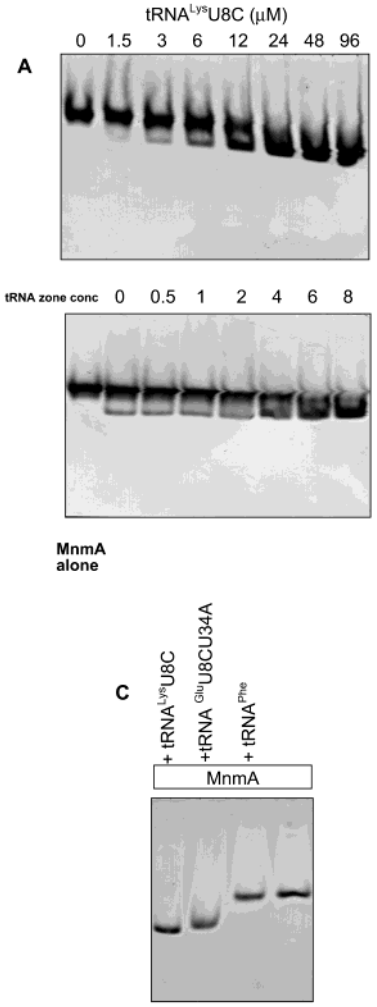

FIGURE 5: Analysis of tRNA binding of MnmA by agarose gel mobility shift and zone-interference electrophoresis. MnmA·tRNA complexes were electrophoresed on 2% vertical agarose gels as described in the Materials and Methods. The gels were visualized by Coomassie staining. Panel A shows the agarose gel mobility shift. MnmA (6.0 μM) was incubated with 0, 1.5, 3, 6, 12, 24, 48, 96 μM tRNA^{Lys}U8C and subjected to electrophoresis. Panels B and C show the zone electrophoresis. Panel B, complexes of MnmA + tRNA^{Lys}U8C (32 μM each, 5 μL, 10% glycerol) were applied beneath zones of tRNA solution (0, 0.5, 1, 2, 4, 6, 8 μM, 50 μL, 4% glycerol) and electrophoresed. Panel C, MnmA (32 μM) was applied beneath zones of either tRNA^{Lys}U8C (64 μM) or tRNA^{Lys}-U8CU34A (12.4 μM) or tRNA^{Phe} (19.4 μM) and electrophoresed.

hands. These methods included nitrocellulose filter binding and gel shift assays using polyacrylamide native gels. Titration of intrinsic MnmA fluorescence by tRNA^{Glu} gave a K_d value of 3 μM (data not shown) but this was only an estimate because of limitations due to the inner filter effect at high tRNA concentrations. The agarose gel shift data allowed us to measure K_d with adequate confidence, since the fraction bound can be observed directly from the gel. Figure 5 shows a series of gel shift experiments. Panel A shows the mobility shift of MnmA with increasing concentrations of tRNA^{Glu}U8C. We used the U8C mutant for consistency with earlier activity studies. This mutant has similar activity in our in vitro assay as the wt tRNA^{Glu}. The data from panel A gives a K_d of approximately 10 μM, by densitometry analysis of the Coomassie stained protein bands. We also used a technique called zone electrophoresis (*30*), in which protein−nucleic acid complexes are added beneath

increasing concentrations of tRNA in each loading well of the gel. This technique is designed to minimize dissociation for weak complexes as they traverse the gel, thus mimicking equilibrium gel filtration methods, and has been applied by Wu et al. to study tRNA binding by glycyl tRNA synthetase (*34*). Using this method, we observe a K_d of 6 μM. Panel C shows zone electrophoresis with excess $tRNA^{Lys}$U8C, $tRNA^{Glu}$-U8C,U34A and nonsubstrate $tRNA^{Phe}$. This data shows an interaction with $tRNA^{Lys}$U8C and the $tRNA^{Glu}$U8C, U34A mutant but no observable mobility shift with $tRNA^{Phe}$, which corroborates the protease protection data. These experiments provide evidence that MnmA binds to unmodified $tRNA^{Glu}$ with an affinity that is physiologically relevant.

Assay for in Vitro s^2U Biosynthesis. We have used either $tRNA^{Glu}$U8C or $tRNA^{Lys}$U8C transcripts as substrates in the assays, to minimize possible formation of s^4U, a modification of the uridine invariably present at position 8 of all bacterial tRNAs. Later work showed that the wt tRNAs have equal substrate activity. Initially, for standardization of assay conditions, we overexpressed MnmA in a *thiI*$^-$ *E. coli* mutant and used S-30 supernatant from cells overproducing MnmA. This avoided presence of ThiI, which catalyzes s^4U synthesis using sulfur mobilized from cysteine by IscS (*25*). Since the tRNA of the *iscS* mutant does not contain the s^2U modification (*44*−*46*), we concluded that IscS is a requirement for s^2U biosynthesis. We thus attempted to reconstitute in vitro s^2U biosynthesis using overexpressed MnmA S-30 supernatant, purified IscS, ATP, Mg^{2+}, PLP, and [^{35}S]-L-cysteine. The reactions were analyzed for ^{35}S incorporation into tRNA using a DEAE filter disk assay (*25*, *31*).

In our hands, the thiolation activity of the reconstituted system was 10-fold over the protein alone control. The specific activity with $tRNA^{Glu}$ was 3 pmol/min/mg S-30 extract. Removal of small molecules from MnmA S-30/S-100 extracts by chromatography on PD-10 columns did not effect the level of activity. To confirm a requirement for IscS in s^2U synthesis, we overexpressed MnmA in the *iscS*$^-$ strain CL100(DE3) and isolated S-30/S-100 extracts. Although we had less expression of MnmA due to the poor growth of the parent *iscS*$^-$ strain, we have found that activity was lost in these extracts (data not shown). No activity was observed for cell extracts isolated from either wild type *E. coli* (BL21 strain) or *mnmA*$^-$ strain. No activity was observed when ATP was replaced with GTP (data not shown).

Identification of 2-Thiouridine as Product of the in Vitro s^2U Reaction. To confirm the presence of s^2U in the in vitro thiolated tRNA, we digested ^{35}S-labeled $tRNA^{Glu}$U8C into nucleosides using nuclease P1 and alkaline phosphatase (*33*) and analyzed the mixture by HPLC (Figure 6). Authentic s^2U elutes at 21.83 min immediately after guanosine (20.60 min) (panel A). The ^{35}S-tRNA digest shows a discernible peak reproducibly at 21.80 min in the chromatogram (panel B), that is consistent with the standard s^2U shown in panel A. We found that 95% of the recovered ^{35}S-label (panel C, fraction 22) comigrated with the peak at 21.80 min shown in panel B. These results confirm that s^2U is the sole thionucleoside present in the in vitro thiolated tRNA.

Kinetic Properties of the in Vitro Reaction. We assayed a number of potential substrates for MnmA catalyzed s^2U formation using the filter disk assay. Because of the low specific activity of MnmA in our hands, large amounts of protein were required to observe s^2U formation. Assays

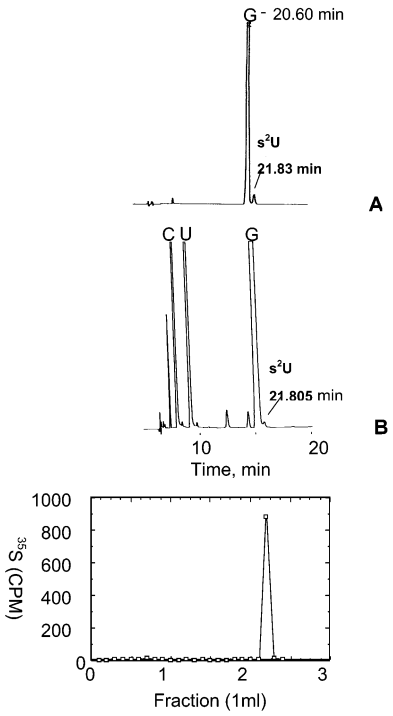

FIGURE 6: HPLC analysis of tRNA thiolated in the in vitro s^2U assay. Panel A shows the separation of s^2U and guanosine standards. Panel B shows the chromatogram of the enzymatically digested tRNA reaction product. Panel C shows the profile of radioactivity (^{35}S) of aliquots taken from the eluent shown in panel B.

containing 5 μM (10 μg) MnmA, 1.5 μM (3.5 μg) IscS, and 5 μM substrate tRNA gave a specific activity of 30−50 pmol/min/mg MnmA. We observed a linear rate of sulfur incorporation over 30 min; however, we were unable to measure accurate kinetic constants because of severe substrate inhibition at tRNA concentrations greater than 5 μM. This inhibition was observed at similar levels for both $tRNA^{Glu}$ and $tRNA^{Lys}$. The level of inhibition for $tRNA^{Glu}$ was 40% at 20 μM and 70% at 40 μM compared to the rate at the optimum concentration of 5 μM. The inhibition was not seen when 20 μM $tRNA^{Phe}$ was added to reactions containing 5 μM $tRNA^{Glu}$. This is consistent with the lack of observed binding to MnmA by $tRNA^{Phe}$. Measurement of kinetic constants at steady state was also compromised by the low specific activity which required enzyme concentration to be on the order of the tRNA substrate. For comparison, the specific activity of ThiI-catalyzed formation of s^4U in $tRNA^{Glu}$ is 150-fold higher (4800 pmol/min/mg ThiI or $k_{cat} = 0.3$ min^{-1}). Substrate inhibition is not observed in this system.

Anticodon Stem Loop Structures Are Substrates for MnmA s^2U Modification. We prepared small RNAs corresponding to the sequences of the anticodon stem loop of $tRNA^{Glu}$ and $tRNA^{Lys}$ (Figure 7). These RNAs were found to be good substrates for MnmA catalyzed s^2U formation (Table 2). The $tRNA^{Lys}$ stem loop (TLYS-ACSL) in particular was modified at the same rate as the full-length $tRNA^{Lys}$ when both are at 5 μM, whereas the Glu derivative (TGLU-ACSL) required higher concentrations for efficient conversion. As shown in Table 2, higher concentrations of TGLU-ACSL gave an increase in activity rather than inhibition. We are currently analyzing these substrates and other variants to gain a more complete picture of both the kinetics and substrate specificity of this system.

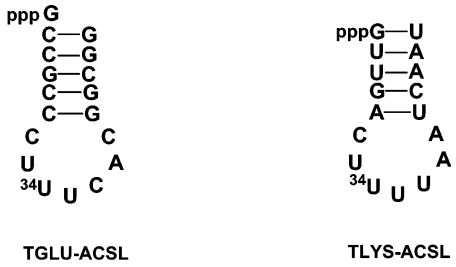

FIGURE 7: Structures of anticodon stem loop (ACSL) RNAs based on $tRNA^{Glu}$ (TGLU-ACSL) and $tRNA^{Lys}$ (TLYS-ACS).

Table 2: Substrate Specificity of MnmA Catalyzed s^2U Modification

substrate (5 μM)	s^2U synthesis (pmol/min/mg MnmA)
wt $tRNA^{Glu}$	33 ± 3
$tRNA^{Glu}$U8C	50 ± 1
$tRNA^{Glu}$U34A	<0.3
wt $tRNA^{Lys}$	41 ± 2
TGLU-ACSL (5 μM)	7 ± 1
TGLU-ACSL (20 μM)	20 ± 3
TLYS-ACSL	38 ± 7

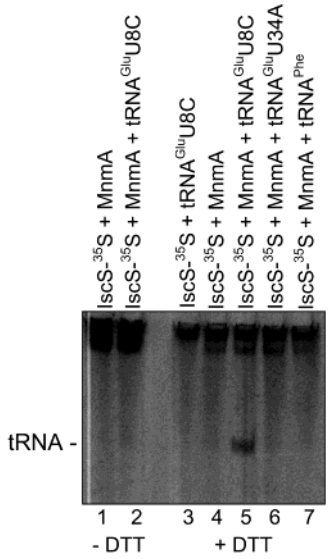

FIGURE 8: MnmA catalyzes thiolation of substrate tRNA transcript using IscS-^{35}S persulfide. Reactions were carried in the presence and absence of DTT as described in the Materials and Methods. Lane 1, IscS-^{35}S persulfide + MnmA; lane 2, IscS-^{35}S persulfide + MnmA + $tRNA^{Lys}$U8C; lane 3, IscS-^{35}S persulfide + $tRNA^{Lys}$U8C; lane 4, IscS-^{35}S persulfide + MnmA; lane 5, IscS-^{35}S persulfide + MnmA + $tRNA^{Lys}$U8C; lane 6, IscS-^{35}S persulfide + MnmA + $tRNA^{Glu}$U34A; lane 7, IscS-^{35}S persulfide + MnmA + $tRNA^{Phe}$.

Isolated IscS-Persulfide Provides Sulfur in the in Vitro s^2U Assay. We isolated IscS-persulfide after reaction of IscS with [^{35}S]cysteine using size-exclusion chromatography on PD-10 columns. Quantitation of ^{35}S-label on the IscS-persulfide revealed that 20% of IscS contained bound sulfur. We then attempted to reconstitute in vitro s^2U biosynthesis using IscS-persulfide in the absence of cysteine (Figure 8). Addition of IscS-^{35}S persulfide to substrate $tRNA^{Glu}$U8C in the presence of Mg-ATP and MnmA resulted in the transfer of label to tRNA (lane 5). Interestingly, we have observed that at least 0.1 mM DTT is required for the transfer of ^{35}S-label from IscS-^{35}S persulfide to tRNA substrate. No labeled tRNA was detected when DTT was omitted in the reaction mixture (lane 2). No transfer of ^{35}S-label from IscS-^{35}S persulfide was observed when nonsubstrate $tRNA^{Glu}$U34A (lane 6) or $tRNA^{Phe}$ (lane 7) was included in the reaction. In addition, in the absence of MnmA, IscS-^{35}S persulfide alone did not transfer ^{35}S-label to tRNA substrate (lane 3). These results show that sulfur in the form of IscS persulfide is catalytically competent to support s^2U biosynthesis in vitro.

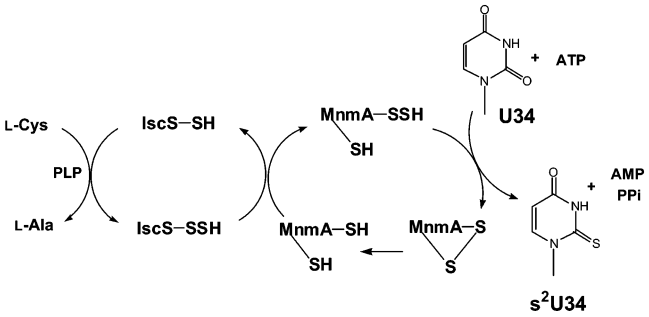

FIGURE 9: Possible mechanistic scheme for s^2U formation catalyzed by IscS and MnmA.

DISCUSSION

We have shown that MnmA binds to an unmodified substrate tRNA with physiologically relevant affinity and catalyzes the synthesis of s^2U in vitro using sulfur mobilized from cysteine by IscS. The in vitro reaction is specific for $tRNA^{Glu}$ and $tRNA^{Lys}$ and requires a uridine at position 34, the site of in vivo modification. The activity of anticodon stem loop structures of the substrate tRNAs suggests that the major determinants for substrate recognition reside in these smaller structures. The reaction overall is very similar to the biosynthesis of s^4U catalyzed in *E. coli* by IscS and ThiI. Both reactions require similar components although the optimal concentrations and, in particular, the specific activity vary. Depending on the organisms compared, there is weak but detectable sequence homology between MnmA and ThiI, mostly in the nucleotide triphosphate binding P-loop region. Both enzymes also show similarity in this region to GMP synthetase and other enzymes that activate substrates by adenylation as has been observed previously (*35*).

A putative reaction scheme (Figure 9) can be proposed based on our results and extrapolation from the similarities to s^4U synthesis. IscS first catalyzes cysteine desulfuration with concomitant formation of a persulfide at Cys-328 in its active site (*36*). We have previously shown that persulfide sulfur can be transferred from IscS to ThiI in the formation of s^4U in vitro (*37*). If the analogy with s^4U synthesis holds, the persulfide sulfur is transferred from IscS to a cysteine on MnmA. The 2-position of U34 in substrate tRNA is most likely activated by adenylation with Mg-ATP by MnmA, which could transfer a persulfide sulfur via mechanisms outlined for s^4U synthesis by us (*37*) and more specifically by Mueller et al. (*38*). The final products are then s^2U, AMP, PPi, and oxidized MnmA.

Mueller et al. (*38*) have recently provided evidence for the oxidation of ThiI after single turnover in the formation of s^4U. Two cysteines, C456 and C344, in ThiI are observed to be crucial for s^4U formation. In support of this mechanism in s^2U synthesis, we find that *E. coli* MnmA contains seven cysteine residues, many of which are highly conserved. Many of these are also required in vivo (C. Lauhon, unpublished).

While we have shown previously that ThiI-persulfide formation lies on the reaction pathway for s^4U synthesis in vitro (*37*), our attempts at observing a MnmA-persulfide are inconclusive at present. However, we've provided evidence that purified IscS persulfide is a competent source of sulfur for s^2U synthesis in the presence of DTT (Figure 8). The concentration of IscS persulfide utilized ($\sim$1 μM), is physiologically relevant given that the in vivo concentration of IscS in *E. coli* has been estimated at 45 μM (*47*). Xi et al. (*39*), have recently provided evidence for a novel mechanism for IscS persulfide in thiazole biosynthesis that involves direct attack of the persulfide on a protein C-terminal adenylate. Although we find no evidence for tRNA binding by IscS alone (*37*), we cannot rule out the formation of a ternary complex that involves initial attack of IscS persulfide on the activated uridine. Current efforts are directed at elucidating the nature of the stepwise transfer of sulfur in the reaction.

One observation that bears comment is the lower specific activity of IscS/MnmA catalyzed s^2U synthesis in vitro compared to IscS/ThiI catalyzed s^4U synthesis. We found the specific activity for MnmA with $tRNA^{Glu}$ as a substrate (0.033 nmol/min/mg MnmA) to be over 100-fold less than ThiI-catalyzed formation of s^4U in $tRNA^{Phe}$ (4.8 nmol/min/mg ThiI) under conditions where each is rate limiting. Numerous attempts at optimization of the assay have led only to modest improvement. We have used unmodified transcripts as substrates in our assay. We found that unmodified transcripts of both $tRNA^{Glu}$ and $tRNA^{Lys}$ gave two bands on native gels, both of which are active as substrates for s^2U synthesis. This observation is consistent with studies that indicate modifications are important for *E. coli* $tRNA^{Glu}$ tertiary structure (*7*). It is conceivable that base modifications are required for optimal substrate binding and activity. However, since the anticodon stem loops appear to be good substrates, there are no common modified bases between $tRNA^{Glu}$, $tRNA^{Lys}$ and $tRNA^{Gln}$ other than the 5-methylaminomethyl group at U34, which is not necessary for s^2U formation in vivo (*15*, *18*, *21*). Nonetheless, recent results of Sunderam et al. (*40*) have shown that hypermodification of U34 and of A37 result in conformational preferences within the anticodon loop. We are currently evaluating hypomodified variants of substrate tRNAs expressed in our *mnmA* mutant to assess the effect of the other modifications.

There is another possible explanation for the lower activity of MnmA. ThiI from *E. coli* and a few other Gram-negative organisms contains a C-terminal domain of about 100 amino acids that has homology to rhodanese-like sulfurtransferases (*41)*. Cys-456 present in this extension is shown to be critical for s^4U synthesis in *E. coli* (*41*), but the extension is absent in the *thiI* genes of most organisms and is absent in *mnmA* and its homologues. In *Bacillus subtilis*, which has a truncated *thiI*, s^4U levels are significantly lower than in *E. coli* (*42*). We have analyzed tRNA from *B. subtilis* mutants lacking a *thiI* homologue (*ytbJ*) and find no s^4U.[2] Thus, this gene is required for s^4U synthesis in vivo. It is not known whether the truncated ThiI from these organisms is active with IscS alone, or if there are sulfurtransferases present that can act in trans to stimulate the reaction. Many putative rhodanese-like sulfurtransferases exist in these organisms (*43*), yet their biochemical roles remain unknown. We have analyzed the tRNA from an *E. coli* mutant that lacks seven of these rhodanese homology genes.[3] We find that this heptuple mutant contains wild type levels of each of the four naturally occurring thionucleosides. Attempts to identify factors in cell extracts that stimulate the in vitro reaction have been unsuccessful. The possibility remains, however, that the specific activity observed in vitro is sufficient to maintain the observed levels of s^2U in vivo.

[2] Lauhon, C. T., unpublished material. The *B. subtilis ytbJ* mutant was a generous gift from S. Aymerich.

[3] Donahue, J. L., Ahmed, F., Larson, T. J., Lauhon, C. T., unpublished material.

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